Isotope Effect Studies on the Calcineurin Phosphoryl-Transfer Reaction: Transition State Structure and Effect of Calmodulin and Mn^{2+} †

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ABSTRACT: The hydrolysis of *p*-nitrophenyl phosphate (*p*NPP) catalyzed by calcineurin has been studied by measurement of heavy-atom isotope effects in the substrate. The isotope effects were measured at the nonbridging oxygen atoms [$^{18}(V/K)_{nonbridge}$], at the position of bond cleavage in the bridging oxygen atom [$^{18}(V/K)_{bridge}$], and at the nitrogen atom in the nitrophenol leaving group [$^{15}(V/K)$]. The isotope effects increased in magnitude upon moving from the pH optimum of 7.0 to 8.5; $^{18}(V/K)_{bridge}$ increased from 1.0072 to 1.0115, and $^{15}(V/K)$ from 1.0006 to 1.0014. The value for $^{18}(V/K)_{nonbridge}$ is 0.9942 at pH 8.5. These data are consistent with P−O bond cleavage being partially rate-limiting at the pH optimum and more so at the higher pH. The $^{18}(V/K)_{nonbridge}$ isotope effect indicates that the dianion is the substrate for catalysis, and a dissociative transition state is operative for phosphoryl transfer. Increasing the concentration of the activating metal ion Mn^{2+} at pH 7.0 from 1 mM to 5 mM increases the magnitude of the isotope effects by an amount similar to that observed with the shift in pH from 7.0 to 8.5, indicative of a change in the commitment factor in the kinetic mechanism so as to make the chemical step more rate-limiting.

The regulation of many biological processes is accomplished by the formation and cleavage of phosphate esters, which is balanced by the interplay of protein kinases and phosphatases. Protein phosphatases are broadly classified by target specificity into the serine/threonine phosphatases, the tyrosine-specific phosphatases, and the dual-specific phosphatases. There are also nonspecific phosphatases which are active on phosphoproteins. Understanding of the catalytic mechanism of the serine/threonine protein phosphatases trails the understanding of protein-tyrosine phosphatases (PTPases) and the nonspecific phosphatases. The PTPases and the nonspecific phosphatases (with one significant exception) seem to all catalyze the hydrolysis of the phosphate ester bond by formation of a phosphoryl-enzyme intermediate, although different amino acids are utilized by different enzymes. PTPases generate a phosphoryl-cysteine intermediate (*1*,*2*); nonspecific alkaline phosphatases (*3*) form phosphoryl-serine intermediates; and nonspecific acid phosphatases (*4*) generate either phosphoryl-histidine or phosphoryl-cysteine as an intermediate. In contrast, there is no positive evidence yet for the formation of a covalent intermediate by a serine/threonine protein phosphatase. Indeed, the preponderance of evidence indicates that these enzymes catalyze the direct transfer of the phosphoryl group to water. Such a distinction may be a critical difference for the regulation of these classes of phosphatases.

The serine/threonine phosphatases are distinguished from the PTPases and the dual-specific protein phosphatases by utilizing two-metal-ion catalysis. There are four major protein serine/threonine phosphatase classes (*5*, *6*) distinguished primarily by substrate specificities and susceptibility to specific phosphatase inhibitors; the phosphatases are designated types-1, -2A, -2B, and -2C. Phosphatases-1, -2A, and -2B share extensive sequence identity and are considered to belong to a single gene family (*7*). Recently crystal structures of both PP-1 (*8*, *9*) and calcineurin (PP-2B) (*10*, *11*) have been obtained. These enzymes have very similar active sites, and these structures have confirmed the notion that serine/threonine phosphatases bind two metal ions in the active site.

Calcineurin is of considerable interest because of its essential role in the T cell activation pathway and its calmodulin dependence, indicating unique functions among this family of phosphatases (*12*, *13*). In order to study the mechanism and transition state structure of the phosphoryl-transfer reaction catalyzed by calcineurin and related enzymes, we have measured heavy-atom isotope effects for the calcineurin-catalyzed hydrolysis of *p*-nitrophenyl phosphate (*p*NPP). A detailed description of the transition state structures involved in reactions can be obtained from kinetic isotope effects. In a phosphoryl-transfer reaction, the secondary ^{18}O isotope effect in the nonbridge oxygen atoms, $^{18}(V/K)_{nonbridge}$, reveals whether the transferring phosphoryl group in the transition state is metaphosphate-like, as expected in a dissociative mechanism, or if it resembles a pentavalent phosphorane as expected in an associative

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[1] Abbreviations: CaN, calcineurin; CaM, calmodulin; *p*NPP, *p*-nitrophenyl phosphate; PTPase, protein-tyrosine phosphatase; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; MOPS, 4-morpholinepropanesulfonic acid. In the notation used to express isotope effects, the leading superscript of the heavier isotope is used to indicate the isotope effect on the following kinetic quantity; for example, $^{15}k_3$ denotes $^{14}k_3/^{15}k_3$, the nitrogen-15 isotope effect on the rate constant k_3.

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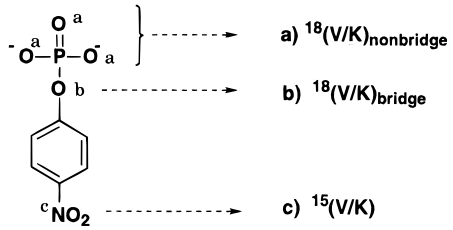


FIGURE 1: Structure of the *p*NPP substrate indicating the atoms where isotope effects were measured in this study.

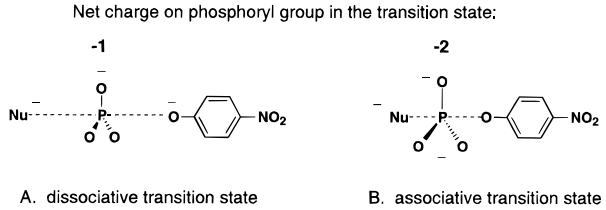


FIGURE 2: Diagram illustrating the difference between a dissociative versus an associative transition state for phosphoryl transfer. In the transition state for a dissociative mechanism, the transferring phosphoryl group bears a single negative charge. In the associative extreme, the phosphoryl group carries a charge of −2 in the transition state. The exact bond order of the P−O bonds in the phosphoryl group in the transition state is uncertain; for simplicity, they are represented here as single bonds.

mechanism. The primary ^{18}O isotope effect in the bridging oxygen atom, $^{18}(V/K)_{bridge}$, is at the site of P−O bond cleavage and gives a measure of the degree of cleavage of this bond in the transition state. When *p*-nitrophenyl phosphate is the substrate, the ^{15}N isotope effect in the leaving group, $^{15}(V/K)$, is sensitive to the amount of negative charge delocalized into the aromatic ring, thus giving a measure of the charge developed on the leaving group in the transition state (*14*). The positions of these three sites of isotope effect measurement in the substrate are shown graphically in Figure 1. All three of these isotope effects have previously been measured for the well-characterized solution hydrolysis reactions of the *p*NPP monoanion and dianion (*15*) as well as for two members of the tyrosine-phosphatase family, the *Yersinia* enzyme and rat PTP1 (*16*), and for the dual-specific phosphatase VHR (*17*). These data provide a background to interpret the isotope effects obtained in this study from the reactions with calcineurin, and to compare the mechanisms and transition states involved.

There is considerable interest in the details of enzymatic catalysis of the hydrolysis of phosphate monoesters, and whether the mechanisms and transition states involved are the same or different from uncatalyzed phosphoryl-transfer reactions in solution. Phosphate monoesters in solution hydrolyze via a concerted S_N2 mechanism (A_ND_N in the IUPAC nomenclature). The transition state is highly dissociative with bond cleavage to the leaving group nearly complete and with a very small degree of bond formation to the nucleophile as illustrated in structure A in Figure 2 (*18*, *19*). Enzymatic phosphoryl transfers of phosphate monoesters which have been studied have generally been found to have a similar mechanism (*16*, *17*, *20*, *21*, *22*, *23*). However, it has been proposed that enzymatic phosphoryl-transfer reactions may prefer an associative mechanism like structure B in Figure 2 because the resulting increased negative charge on the phosphoryl group could be stabilized by catalytic groups (*24*, *25*). One can postulate that strong interactions with metal ions by the nonbridging oxygen atoms could bring about a change to a more associative mechanism such as that resulting from alkyl substitution; phosphodiesters have much more associative transition states than monoesters (*19*). For these reasons, the mechanisms and transition state structures of enzymatic phosphoryl transfers are an area of much current interest.

EXPERIMENTAL PROCEDURES

Materials. EGTA, DEAE-Sepharose, and phenyl-Sepharose were purchased from Sigma. Alkaline phosphatase from *Escherichia coli*, type III, and unlabeled *p*NPP were purchased from Sigma. Diethyl ether used in extractions was distilled before use. [^{14}N]-*p*-Nitrophenol, [^{14}N]-*p*-nitrophenyl phosphate, [^{15}N, *bridge*-^{18}O]-*p*-nitrophenyl phosphate, and [^{15}N, *nonbridge*-$^{18}O_3$]-*p*-nitrophenyl phosphate were synthesized and purified as previously described (*15*). Bovine brain was obtained from Pel-Freez. Solutions, including those used in the preparation of calcineurin, were prepared from water that had been passed over a Chelex-100 column. The MOPS buffer used in the assays was likewise passed over Chelex-100 after preparation of the solution. The pH was checked after passing over Chelex-100 and adjusted to 7.0 before use in assays.

Protein Purification. Calcineurin was isolated from bovine brain by the method of Sharma et al. (*26*) except MOPS was substituted for Tris. Calmodulin was purified by the procedure of Sharma and Wang (*27*) with a slight modification. Following DEAE-chromatography, the calmodulin fraction was loaded onto phenyl-Sepharose and eluted with EGTA (*28*). Protein concentrations were determined by the method of Bradford (*29*).

General Method for Isotope Effect Measurements. The isotope effects in this study were measured by the competitive method, using an isotope ratio mass spectrometer. The ^{18}O isotope effects were measured by the remote label method (*30*). In this technique, substrate is synthesized with labels at two positions, one at the site of chemical interest and the other (the remote label) at a position which lends itself to facile isolation and isotopic measurement. This double-labeled material is mixed with substrate containing only the natural abundance of ^{18}O in the position of interest, but depleted material in the remote label position (see Figure 3). The mixing ratio is such that the natural abundance of ^{15}N is restored in the remote label position. When this mixture is used in an experiment, the observed isotope effect is the product of that in the position of interest and that in the remote label position. The isotope effect in the latter position is determined with substrate containing only the natural abundance of isotopes in all positions, and the ratio of the two observed isotope effects is the desired one in the position of interest. With *p*NPP, the nitrogen atom serves as a convenient remote label which is easily isolated as molecular nitrogen, as previously described (*15*).

Kinetic Isotope Effect Measurements. The isotope effect experiments were performed at 37 °C in buffered solutions (0.1 M) of MOPS (pH 7.0) or TRICINE (pH 8.5) containing 1 mM or 5 mM $MnCl_2$. For measurement of each isotope effect, reactions were started with approximately 100 μmol of *p*NPP substrate. After the solutions were equilibrated at 37 °C, sufficient enzyme was added in order to give from 15% to 30% conversion to product within 72 h. At pH 7.0, reactions were initiated with 150 μL of CaN (4.6 mg/mL)

Table 1: Kinetic Isotope Effects on Reactions of Calcineurin with *p*NPP

conditions, pH	$^{15}(V/K)$	$^{18}(V/K)_{bridge}$	$^{18}(V/K)_{nonbridge}$[a]
Calcineurin Reactions			
5× CaM, 1 mM Mn, pH 7	1.0006 ± 0.0005	1.0072 ± 0.0011	
5× CaM, 1 mM Mn, pH 8.5	1.0014 ± 0.0001	1.0115 ± 0.0012	0.9942 ± 0.0007
no CaM, 1 mM Mn, pH 7		1.0063 ± 0.0018	
no CaM, 5 mM Mn, pH 7		1.0109 ± 0.0009	
5× CaM, 5 mM Mn, pH 7	1.0011 ± 0.0003	1.0103 ± 0.0014	
Comparison Reactions			
reaction	$^{15}(V/K)$	$^{18}(V/K)_{bridge}$	$^{18}(V/K)_{nonbridge}$[a]
aqueous hydrolysis of monoanion[b]	1.0005 ± 0.0002	1.0106 ± 0.0003	1.0224 ± 0.0005
aqueous hydrolysis of dianion[b]	1.0034 ± 0.0002	1.0230 ± 0.0005	0.9993 ± 0.0007
Yersinia PTPase[c]	0.9999 ± 0.0003	1.0142 ± 0.0004	0.9981 ± 0.0015

[a] The isotope effect due to ^{18}O in all three nonbridge oxygen positions. [b] Data from ref *15*. [c] Data from ref *16*.

and 75 μL of CaM (9 mg/mL). Control experiments determined that the uncatalyzed hydrolysis of *p*NPP accounted for 5% or less of the total reaction over the longest reaction time used. In reactions in which CaM was omitted, additional CaN was used to offset the reduced enzymatic rate. For each isotope effect, several reactions were allowed to proceed to varying fractions of reaction. Reaction progress was followed by monitoring the appearance of *p*-nitrophenol from the absorbance at 400 nm of an aliquot of the reaction mixture added to 0.2 N NaOH. When approximately the desired fraction of reaction was reached, an aliquot was removed for determination of the exact fraction of reaction. The reaction progress was stopped by chilling the flasks in ice, followed shortly by titration to pH 5 and extraction of the *p*-nitrophenol with diethyl ether (3 × 25 mL), which leaves unreacted *p*NPP in the aqueous layer. After the ether extracts were dried over magnesium sulfate, the ether was removed by rotary evaporation. The aliquot which was removed prior to ether extraction was diluted into Tris buffer at pH 9 and used to determine the fraction of reaction by assaying for free *p*-nitrophenol at 400 nM, treating with alkaline phosphatase overnight, and then reassaying at 400 nm.

The unreacted *p*NPP substrate in the aqueous layer was completely hydrolyzed by treatment with alkaline phosphatase (3 units) at pH 9 in Tris buffer which was 1 mM in both Zn and Mg. The *p*-nitrophenol thus produced was isolated by acidification to pH 5 and ether extraction, as for the initial product. The *p*-nitrophenol samples from both product and residual substrate at partial reaction were further purified by sublimation, and then combusted to produce nitrogen gas which was isolated and analyzed by isotope ratio mass spectrometry as previously described (*15*).

Isotope effects were calculated from the isotopic ratio of nitrogen in the *p*-nitrophenol product at partial reaction (R_p), in the residual substrate (R_s), and in the starting material (R_o). Equation 1 was used to calculate the observed isotope effect from product and starting material at fraction of reaction *f*, and eq 2 was used to calculate the observed isotope effect from residual substrate and starting material. Thus, each experiment yields two independent measurements of the isotope effect.

$$\text{isotope effect} = \log (1 - f)/\{\log [1 - f(R_p/R_o)]\} \quad (1)$$

$$\text{isotope effect} = \log (1 - f)/\{\log [(1 - f)(R_s/R_o)]\} \quad (2)$$

At least four independent determinations were made of each isotope effect. The results calculated from R_p and R_o, and from R_s and R_o, were within experimental error of one another in all cases, as were those measured at differing fractions of reaction. The observed ^{18}O isotope effects from these experiments were corrected for the ^{15}N effect and for incomplete levels of isotopic incorporation in the starting material (*31*).

The isotopic ratio of nitrogen in the starting material, R_o, was determined both by direct combustion of samples of *p*NPP substrate and, as a control, by completely hydrolyzing samples of *p*NPP and analyzing the liberated nitrophenol following isolation by the same method used in the isotope effect experiments. The isotopic ratios obtained from both methods were the same within experimental error, showing that no isotopic fractionation occurs during the procedures used to recover *p*-nitrophenol.

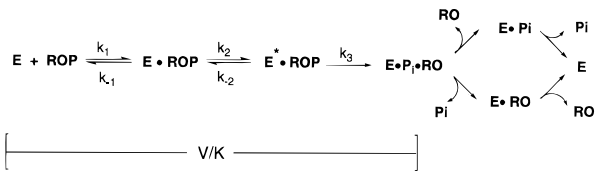
RESULTS

The isotope effects for the CaN reaction with *p*NPP are listed in Table 1 with their standard errors. The isotope effects were measured at the optimal pH of 7.0, and also at pH 8.5 where chemistry is slower (*32*) and thus possibly more rate-limiting. These experiments were run with a 5-fold molar excess of CaM to give maximal activation (*33*), and 1 mM Mn^{2+}. In order to test the effect of levels of CaM and Mn^{2+} on the kinetic mechanism and on the transition state structure, isotope effects were measured at pH 7.0 in the absence of calmodulin, and separately with the usual 5-fold molar excess of CaM and 5 mM Mn^{2+}. Attempts to use the higher 5 mM Mn^{2+} concentration at pH 8.5 resulted in the formation of precipitate.

Hydrolysis of the dianion of phosphate esters showed higher catalytic turnover than the monoanion (*32*), but seemed to have lower affinity (K_m increased with pH). Isotopic fractionation in the nonbridge oxygen atoms affects the proportion of isotopic isomers present as the dianion at a given pH which could potentially affect the observed value for $^{18}(V/K)_{nonbridge}$. However, because the pK_a of *p*NPP is 4.96 (*34*), at the lowest pH value used in these experiments the substrate is >99% deprotonated. Thus, no fractionation in formation of the dianion will occur under these conditions, and $^{18}(V/K)_{nonbridge}$ will be unaffected.

For comparison with the calcineurin data, the isotope effects for the uncatalyzed hydrolysis in solution of *p*NPP, which were measured in previous work, have been included in Table 1 as well as the data for the PTPase from *Yersinia*. The *Yersinia* PTPase data are very similar to the results

Scheme 1



obtained from the PTPase PTP1 and to those from the human dual-specificity phosphatase VHR.

DISCUSSION

Factors Influencing the Isotope Effects. A simplified version of the calcineurin mechanism is shown in Scheme 1. The isotope effects in this study were measured by the competitive method and therefore yield isotope effects on *V/K* (*35*) and are thus sensitive only to steps from substrate binding up to and including the first irreversible step. The kinetic sequence of binding of metal ions to CaN and to CaM, and binding of CaM to CaN, is not known. It is assumed that under the conditions used, binding of Mn and CaM to calcineurin will be complete and precede binding of *p*NPP, due to the high K_m for *p*NPP. Any such binding steps or conformational changes that occur before binding of *p*NPP will have no effect on the isotope effects and thus are omitted from Scheme 1. The scheme includes a nonchemical step, k_2, which represents a hypothetical conformational change or other nonchemical step following binding of *p*NPP and preceding catalysis. The chemical step of phosphoryl transfer is assumed to be irreversible in Scheme 1. The justifications for this are the absence of phosphotransferase activity (*33*), the failure to demonstrate isotope exchange between phosphate and water (*36*), the poor nucleophilicity of nitrophenol, and the high K_i and rapid release of nitrophenol from the active site (*32*).

The interpretation of kinetic isotope effects on an enzymatic reaction depends upon knowledge of the degree to which the chemical step is rate-limiting. To the degree that a nonchemical step such as substrate binding or a conformational change is partially or fully rate-limiting, the intrinsic isotope effects on the chemical step will be suppressed or abolished completely, and will not reflect the transition state structure. If only the phosphoryl-transfer step (k_3) in *V/K* is isotope-sensitive, the observed isotope effect will be given by eq 3 (*37*). In this equation, $^*(V/K)$ represents the isotope effect on *V/K*, and $^*k$ similarly designates the isotope effect on the isotope-sensitive step. The commitment factor c_f is equal to $(k_3/k_{-2})(1 + k_2/k_{-1})$.

$$^*(V/K) = (^*k_3 + c_f)/(1 + c_f) \qquad (3)$$

The ratio k_2/k_{-1} reflects the fate of the initial enzyme−substrate complex. If the *p*NPP substrate is "sticky" (k_2/k_{-1} is large), the isotope effect on the subsequent chemical step will be suppressed. The fact that *p*NPP is a slow substrate for calcineurin with a relatively high K_m in the millimolar range (*33*) indicates that the ratio k_2/k_{-1} should be small. The ratio k_3/k_{-2} reflects the fate of the enzyme−substrate complex after the hypothetical nonchemical step. If such a step, for example, a conformational change, is not rapidly reversible, then the *p*NPP substrate will be committed to undergo phosphoryl transfer, and the large value for k_3/k_{-2} will suppress the observed isotope effect.

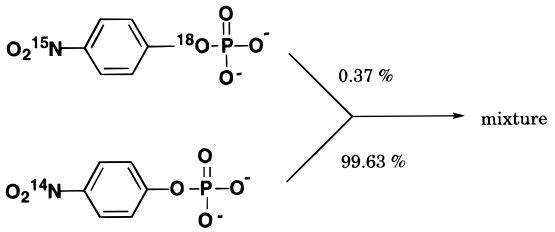


FIGURE 3: Diagrammatic representation of the use of the remote label method to measure the ^{18}O isotope effect in the bridging position, $^{18}(V/K)_{bridge}$. Each molecule bearing an atom of ^{18}O in the position of interest also is labeled with ^{15}N. Thus, the isotopic fractionation of nitrogen will act as a reporter for isotopic fractionation at the oxygen position. The two labeled versions of the substrate are mixed in the proportions shown, and the mixture is used as the substrate in the competitive method (*38b*).

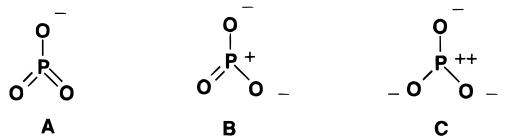


FIGURE 4: Resonance structures for the metaphosphate ion. Though traditionally represented as A, computational studies and isotope effect results indicate metaphosphate exists primarily as a hybrid of structures B and C.

One method for determining whether the chemical step in an enzymatic reaction is rate-limiting and the isotope effects are the intrinsic ones for the chemical step is to compare the enzymatic isotope effects with those for the corresponding uncatalyzed reaction in solution, because the latter reaction is not subject to possible suppression of isotope effects which can occur in enzymatic reactions. Another technique is to determine the enzymatic isotope effects at the pH optimum for reaction, and also at a pH well off the optimum where chemistry is slower and thus possibly more rate-limiting. A large commitment factor will completely suppress isotope effects, yielding observed effects of unity even at pH values well away from the optimum. This is observed in the reaction of alkaline phosphatase with *p*NPP (*15*). This indicates that a nonchemical step occurring after substrate binding is completely rate-limiting for *V/K*. A smaller commitment factor will suppress but not abolish isotope effects on the chemical step. Movement to a pH well off the optimum may then serve to reduce the commitment and increase the magnitudes of the observed isotope effects. In such cases, the limiting values of the isotope effects at the nonoptimal pH will usually still not reflect the full intrinsic isotope effects on the chemical step (*38a*). Only if commitment factors are negligible will the full intrinsic isotope effects be expressed at the pH optimum, and these values will then not change with pH.

The isotope effects on the uncatalyzed hydrolysis of *p*NPP are a useful yardstick for interpreting the effects with calcineurin. In the uncatalyzed reaction of the dianion, the leaving group departs as the anion, and since the transition state for this reaction is highly dissociative, the isotope effects represent essentially complete cleavage of the P−O bond, and ^{15}k represents delocalization of nearly a full negative charge into the aromatic ring. The near-unity value for $^{18}k_{nonbridge}$ is consistent with computational results suggesting that metaphosphate should not be portrayed as is traditionally done, as structure A in Figure 4, but more properly as a hybrid of structures B and C, with no increase in P−O bond order in the metaphosphate-like transition state. In the

monoanion reaction, proton transfer to the leaving group occurs concurrently with P−O bond cleavage. This results in a decreased value for $^{18}k_{bridge}$ because P−O bond cleavage is partially compensated for by O−H bond formation in the transition state. The ^{15}k value is nearly abolished because the leaving group now departs as a nearly neutral species with no charge in the aromatic ring. In the monoanion reaction, the large $^{18}k_{nonbridge}$ effect arises from deprotonation of the phosphoryl group in the transition state.

While the assumption that only the phosphoryl transfer step will be isotope-sensitive is reasonable for the isotope effects in the leaving group, it is possible that the binding step will be isotope-sensitive for the nonbridge oxygen atoms due to interaction between one or more of these atoms with either or both the Zn^{2+} or Fe^{3+} ions at the active site. A crystal structure of bovine CaN with phosphate bound at the active site shows the phosphate bound in bidentate fashion between the two metal ions (*10*), but this does not give information as to the extent or fashion of binding of the phosphoryl group of substrate during catalysis. If binding is isotope-sensitive, then the observed $^{18}(V/K)_{nonbridge}$ isotope effect will be the product of the equilibrium isotope effect on binding and the kinetic effect for phosphoryl transfer. Complexation of Mg^{2+} with phosphate has no measurable ^{18}O isotope effect (*21*), consistent with a fairly weak and purely electrostatic interaction which does not cause changes in the force constants of the nonbridge P−O bonds. By contrast, formation of Co^{3+} complexes with *p*NPP, which are stronger and have significant covalent character, result in substantial isotope effects (*39*). Whether or not the complexation of phosphate with Zn^{2+}, Fe^{3+}, or Mn^{2+} is isotope-sensitive has not been directly determined. Measurement of the isotope effects on the uncatalyzed hydrolysis of the *p*NPP dianion complexed with these metals was not possible due to the low solubility of these metals at the pHs (>9) necessary to observe reaction of the dianion free from significant contribution of the much more reactive *p*NPP monoanion. Linear free energy relationships on the catalysis by Mg^{2+} and Ca^{2+} of the reaction of *p*NPP with substituted pyridines indicate these metals do not increase the associative character of the transition state (*40*).

In summary, the leaving group isotope effects can give information about the extent of bond cleavage in the transition state, and whether proton transfer to the leaving group accompanies bond cleavage. The nonbridge oxygen isotope effects give information as to whether the transferring phosphoryl group resembles metaphosphate in the transition state, as well as information as to the strength of the interaction between the phosphoryl group and the metal ions during catalysis. The variation of the isotope effects with pH and with levels of CaM or Mn^{2+} can give information about the effects of the levels of these regulators on the commitment factors in the kinetic mechanism.

Conclusions from the Isotope Effects. (A) Nature of the Substrate. Kinetic studies with *p*NPP had led to the tentative conclusion that the dianion is the catalytically active species (*32*). If the monoanion were the substrate, the observed $^{18}(V/K)_{nonbridge}$ would then reflect the equilibrium isotope effect for protonation of the substrate because at the pHs used in these experiments *p*NPP is present essentially quantitatively as the dianion. Protonation of phosphate monoesters results in an inverse isotope effect of 0.9848 at 27 °C *(41)*. The small inverse value of $^{18}(V/K)_{nonbridge}$ measured for the calcineurin reaction is of the magnitude expected for a dissociative transition state and is much smaller than that which would arise from formation of the monoanion of *p*NPP. The data are therefore consistent with the conclusion that the substrate for catalysis is the dianion.

(B) Transition State Structure and Mechanism. The isotope effect having the largest magnitude is $^{18}(V/K)_{bridge}$ and it will be the most sensitive to changes in commitment factors. Therefore, it was measured both at the optimum pH of 7.0 and at pH 8.5 where *V/K* is significantly reduced. This isotope effect increases from 1.0072 at pH 7.0 to 1.0115 at pH 8.5, suggesting that chemistry is only partially rate-limiting at the pH optimum and more so at pH 8.5. The $^{15}(V/K)$ increases by roughly the same proportion (taking the standard errors into account), from 1.0006 to 1.0014. This is consistent with a changing commitment factor which will affect all the isotope effects by the same proportion. By contrast, a change in the transition state structure would result in unproportional changes in the isotope effects reflecting the altered bonding in the transition state.

The significant $^{15}(V/K)$ effect indicates the presence of at least a partial negative charge on the leaving group in the transition state. The two mechanistic extremes with respect to proton transfer to the leaving group are (a) full charge neutralization of the leaving group, as in the aqueous monoanion hydrolysis and the PTPase reactions, and (b) the leaving group departs as the anion, as in the aqueous dianion reaction and in reactions of PTPase mutants in which the general acid has been mutated. The first case is characterized by very small or unity values for $^{15}(V/K)$, and by $^{18}(V/K)_{bridge}$ values of about 1.01. Leaving group departure as the *p*-nitrophenolate anion results in $^{15}(V/K)$ effects of from 1.002 to 1.003, and by $^{18}(V/K)_{bridge}$ values of from 1.02 to 1.03.

It cannot be certain whether chemistry is fully rate-limiting at pH 8.5 in the CaN reaction with *p*NPP, and thus the isotope effects at this pH may not be fully expressed. If it is assumed that chemistry is still only about half rate-limiting at pH 8.5, then the intrinsic isotope effects will be about twice the observed values and would be consistent with leaving group departure as the unprotonated anion. However, pH−rate data (*32*) are indicative of general acid protonation in the CaN reaction. The Bronsted β_{lg} value of −0.34 (*33*, *42*) has also been interpreted as indicating proton transfer to the leaving group (for comparison, β_{lg} is −0.27 for uncatalyzed hydrolysis of phosphate ester monoanions, where proton transfer to the leaving group occurs). Bronsted β values will be suppressed in a similar fashion as the isotope effects to the extent that chemistry is not rate-limiting, and thus also may not reflect the calcineurin transition state structure. However, taken together, the kinetic and isotope effect data are consistent with proton transfer to the leaving group during catalysis, though not completely synchronous with P−O bond cleavage in the transition state, resulting in some negative charge residing on the leaving group. This distinguishes CaN from the protein-tyrosine and dual-specific phosphatases in which full charge neutralization of the leaving group occurs in the transition state (*16*, *17*).

There are two possible candidates to serve as a proton donor. First, water coordinated to exogenous metal may provide a proton (*32*). Second, in the crystal structure of bovine CaN, His-151 is in a position which would enable it to function as a proton donor to the leaving group (*10*).

Whether either in fact fulfills this role awaits proof from site-directed mutagenesis or other experiments.

The $^{18}(V/K)_{nonbridge}$ effect at pH 8.5 is 0.9942, which is slightly larger (more inverse) than nonbridge effects measured for the uncatalyzed or for other enzymatic phosphate monoester reactions (see Table 1). An inverse isotope effect at this position indicates increased bond order to one or more of the nonbridge oxygen atoms in the phosphoryl group. In the highly dissociative transition state that is typical of phosphate monoester reactions, the transferring phosphoryl group resembles metaphosphate ion. In the traditional representation of this species, the P−O bond order has increased to 5/3 from 4/3 in the monoester substrate. Computational results have in recent years indicated that this picture is incorrect, and that no such increased bond P−O bond order occurs, metaphosphate being more correctly represented as a hybrid of structures B and C in Figure 4. Isotope effects are consistent with this view. In the aqueous hydrolysis as well as in a number of enzymatic reactions, the nonbridge isotope effects have consistently been much less inverse than would be predicted if the bonding in metaphosphate resembled that in structure A *(43)*. It is thus unlikely that the inverse $^{18}(V/K)_{nonbridge}$ effect arises from increased P−O bond order in the transition state unless the active site environment alters the structure of the metaphosphate-like phosphoryl group in the transition state. The proximity of the metal ions would be expected to have the opposite effect, if any, on the structure of the phosphoryl group. The alternative explanation that the transition state in the CaN reaction is later (more dissociative) is also unlikely since the previously studied reactions are already highly dissociative, characterized by near-complete bond cleavage to the leaving group in the transition state.

A more likely source for the slightly more inverse $^{18}(V/K)_{nonbridge}$ isotope effect is interaction with one or both of the metal ions at the active site. There is precedent for isotope effects in the nonbridge oxygen atoms of a phosphoryl group to arise from interactions with metal ions. While interaction of Mg^{2+} does not give rise to measurable isotope effects (*21*), interactions with Co^{3+} do so (*39*). The complex between *p*NPP and a Co−cyclen complex which forms reversibly in solution gives rise to an equilibrium ^{18}O effect of 0.9919 for formation of the complex. The stronger, stable complexes formed between cobalt ethylenediamine and *p*NPP and between cobalt pentaamine−*p*NPP give rise to isotope effects about twice as large (*39*). For comparison, the ^{18}O effects for protonation of glycerol 3-phosphate and of hydrogen phosphate dianions are 0.9848 and 0.9814, respectively *(41)*. Thus, an interaction between one or both of the active site metal ions and the phosphoryl group would be consistent with the data. On the basis of charge, the Fe^{3+} ion is likely to have a stronger interaction than the divalent metal ion. Whether this interaction occurs upon binding or solely upon catalysis cannot be determined, because the isotope effects on $^{18}(V/K)_{nonbridge}$ will represent a product of equilibrium effects on binding and the kinetic one for the chemical step.

It should be pointed out that there is no evidence in the isotope effect data that the metal ions serve to make the CaN phosphoryl-transfer mechanism more associative in nature, although it has been speculated that metal ions in an enzymatic active site could favor such a transition state (*25*). The loss of nonbridge P−O bond order that occurs upon formation of a phosphorane-like structure gives rise to large normal isotope effects. Phosphotriesters have such transition states, and the alkaline hydrolysis of a diethyl aryl triester exhibits a $^{18}(V/K)_{nonbridge}$ effect of 1.025 (*31*). If the metal ions in CaN were producing an associative transition state, the observed effect would be a product of the inverse effect for metal complexation and the large normal kinetic effect. The overall observed effect would probably be normal, not the inverse effect observed in the CaN reaction.

It is not obvious that simply because the active site contains the two metal ions that a strong interaction with the negatively charged phosphoryl group will occur. The major driving force in the reaction of the dianion is internal electron donation of the negative charge from the nonbridge oxygen atoms. Nature faces a quandary in that some interaction with the phosphoryl group is necessary for proper positioning of the substrate, but the stronger this interaction, the more this driving force for catalysis will be attenuated. The PTPases all have a positively charged arginine, as well as amide hydrogen bonds, which interact with the phosphoryl group, but these interactions have been found to be isotope-insensitive and therefore fairly weak and purely electrostatic in nature (*16*, *17*). Thus, an interaction with the phosphoryl group strong enough to produce a $^{18}(V/K)_{nonbridge}$ isotope effect is not inherently necessary for binding and positioning of the substrate in a phosphatase.

(C) Effect of CaM and Mn^{2+} Levels on the Commitment Factor. Variation in the magnitudes of the isotope effects with levels of CaM or Mn^{2+} provides information about the effect of these ligands on the contribution of chemistry to the rate-limiting step. In Scheme 1, the chemical step of phosphoryl transfer is represented as k_3, and k_2 represents a postulated nonchemical step which follows the binding of the substrate *p*NPP. The k_2 step could represent a conformational change, binding of an additional Mn^{2+} ion, or some other nonchemical event. The exact kinetic sequence of binding CaM, Mn^{2+}, and substrate, and any conformational changes, has not been determined. However, the expression of isotope effects will be affected by any such steps which follow *p*NPP binding, and for simplicity, the possibility of such events has been represented as a single step in Scheme 1.

Experiments with Mn^{2+} as an activator indicate that essentially full activation is achieved at 1 mM concentration of the metal ion *(44)*. At this level of Mn^{2+}, if CaM is omitted from the reaction mixture, the magnitude of $^{18}(V/K)_{bridge}$ decreases slightly from the value of 1.0072 observed with a 5-fold molar excess of CaM to 1.0063 in the absence of CaM. This small change is within the experimental errors of the measurements.

However, variations in the concentration of Mn^{2+} show a greater effect. In the absence of Mn^{2+}, the enzymatic reaction was too slow to measure the isotope effects since buffer hydrolysis predominated. When the Mn^{2+} concentration was increased from 1 mM to 5 mM, the observed value for $^{18}(V/K)_{bridge}$ doubled both in the presence of CaM (to 1.0103) and in its absence (to 1.0109). This suggests that the commitment factor is reduced by the increased Mn^{2+} concentrations in both cases. If k_2 represents a conformational change, these results indicate that the higher Mn^{2+} levels facilitate the reversibility of this event. Since the substrate *p*NPP has a high K_m, it is unlikely to be a sticky substrate, and the ratio k_2/k_{-1} is probably already very small

($k_{-1} \gg k_2$). If the effect of higher metal ion concentrations is to facilitate a conformational change, an increase in k_2 and k_{-2} could significantly decrease k_3/k_{-2} while not significantly affecting the ratio k_2/k_{-1}. The overall rate of reaction for *V/K* is little affected because if two sequential steps are about equally rate-limiting, increasing the rate of one will not greatly affect the overall rate.

Calcineurin contains multiple binding sites for the activating divalent metal ions, and until the exact sequence of events involved in this regulation is understood, all that can be confidently stated is that higher Mn^{2+} concentrations cause a decrease in the commitment factor, resulting in the chemical step becoming more fully rate-limiting for the *V/K* portion of the mechanism.

Conclusions. The chemical step of phosphoryl transfer is partially rate-limiting on *V/K* for the reaction of *p*NPP with CaN, which makes it unique among other phosphatases that have been examined. Protein-tyrosine phosphatases and dual-specific phosphatases exhibit fully rate-limiting chemistry with *p*NPP, while with alkaline phosphatase a nonchemical step is completely rate-limiting for *V/K*. The leaving group departs with at least a partial negative charge in the transition state of the CaN reaction, indicating that proton transfer lags behind P−O bond cleavage in the transition state. The degree to which the chemical step is rate-limiting is unaffected by levels of CaM, but is increased by high levels of Mn^{2+} which suggests that some nonchemical step (or steps) after substrate binding, but before catalysis, is (are) affected. One of the intrinsic metals (Zn^{2+} and Fe^{3+}) or the exogenous metal (Mn^{2+}) may interact with the phosphoryl group in binding or catalysis, in addition to their possible roles in complexation and activation of the nucleophilic water molecule. This interaction is stronger than that between Mg^{2+} and phosphate in solution, but is weaker than that between Co^{3+} and phosphate. The transition state is not made more associative by the metal−phosphate interactions, and remains dissociative.

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